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MINAR is a novel NOTCH-2 interacting protein that regulates NOTCH-2 activation and angiogenesis

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MINAR IS A NOVEL NOTCH-2 INTERACTING PROTEIN THAT REGULATES NOTCH-2 ACTIVATION AND ANGIOGENESIS

by

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ABSTRACT

Angiogenesis, the formation of new vessels, is a highly regulated and complex cellular process, which plays a crucial role in physiological processes such as embryological development and wound healing. Aberrant angiogenesis is a key feature of common human pathologies, including cancer and inflammation. Neurogenic locus notch homology protein 2 (NOTCH2) signaling is an evolutionarily conserved pathway and a major player in regulating angiogenesis. Despite its fundamental involvement in both embryonic development and human diseases, the processes through which the NOTCH pathway modulates angiogenesis are not fully elucidated.

We have identified Major Intrinsically disordered NOTCH2-Associated Receptor (MINAR) as a novel ligand for NOTCH2. The main objectives of this project were to demonstrate the mechanism of association between MINAR with NOTCH2, and its biological importance in angiogenesis. Our findings reveal that MINAR is an intrinsically disordered cell surface receptor, which is highly expressed in endothelial cells and other tissues of human vasculature. The physical association between MINAR and NOTCH2 increases its order and stability, and also reduces the degradation of MINAR. Moreover, we demonstrate that MINAR regulates NOTCH2 activation to inhibit angiogenesis. Taken together, the data suggest that MINAR is a novel ligand of NOTCH2 and a key regulator of angiogenesis.

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LIST OF ABBREVIATIONS

CBF-1 ..... Recombining binding protein suppressor of hairless

EV ..... Empty Vector

FBS ..... Fetal Bovine Serum

GS ..... Gamma Secretase

GSI ..... Gamma Secretase Inhibitor

HEK ..... Human Embryonic Kidney cells

HUVEC ..... Human Umbilical Venous Endothelial Cells

IDP ..... Intrinsically Disordered Protein

MAML1 ..... Mastermind

MINAR ..... Major Intrinsically-disordered Notch-2 Associated Receptor

NICD ..... NOTCH Intracellular Domain

NOTCH ..... Neurogenic locus notch homology protein

PAE ..... Porcine Aortic Endothelial

PVDF ..... Polyvinylidene difluoride

VEGF ..... Vascular Endothelial Growth Factor

VEGFR ..... Vascular Endothelial Growth Factor Receptor

INTRODUCTION

Angiogenesis is the development of new vessels from pre-existing vessels (Folkman, 1996). Physiological angiogenesis largely occurs during the developmental stage in utero, though it continues throughout adulthood during events such as wound healing and menstruation (Papetti, 2002). Angiogenesis could occur in two forms: non-sprouting (intussusceptive) angiogenesis and sprouting angiogenesis (Ribatti et al., 2012). Intussusceptive angiogenesis is the splitting of a single vessel by the growth and formation of interstitial tissue pillars into the lumen, producing a septum that creates two new vessels (Burri et al., 1990; Djonov et al., 2000). Sprouting angiogenesis is the formation of new vessels by branching off from a main vessel. The current model for sprouting angiogenesis involves of two main cell types, tip and stalk cells (Kurz et al., 1996). Tip cells are migratory and distinguished from other endothelial cells as being highly polar, possessing long filopodia, and non-proliferating (Gerhardt et al., 2003). In contrast, stalk cells are highly proliferating and form tight and adherent junctions to support the lumen of the newly sprouting vessel (Dejana et al., 2009; Gerhardt et al., 2009).

Angiogenesis is governed by complex cross-talks between pro-angiogenic and anti-angiogenic factors that coordinate the dynamic equilibrium of cell migration, cell proliferation, and cell survival (Rousseau, 1997; Adams, 2007; Adair, 2010). Although various proteins are involved in the regulation of angiogenesis, three main pathways involved in the regulation of angiogenesis have been identified and include the vascular

endothelial growth factor (VEGF) pathway, Ephrin (Eph) pathway, and NOTCH pathway.

Role of VEGF in angiogenesis

A) VEGF ligands

VEGF, which was originally identified as Vascular Permeability Factor (VPF), is the most prominent player in sprouting angiogenesis (Hoeben et al., 2004). The VEGF ligands family consists of seven forms (VEGF A-F and Placental Growth Factor (PlGF)). Among all the VEGF ligands, VEGF-A is the best-characterized potent inducer of sprouting angiogenesis (Ferrara et al., 2003). Release of VEGF-A in response to angiogenic stimuli, such as hypoxia (*i.e*., low oxygen), is responsible for initiating tip cell selection, the first step in sprouting angiogenesis, where VEGF-A triggers differentiation in a single endothelial cell by binding to and activating VEGF receptor-2 (VEGFR-2) (Gerhardt et al., 2003; Krock et al., 2011). The spatial concentration gradient of VEGF-A is critical in providing directional guidance and extension of filopodia in tip cells (Ruhrberg et al., 2002; Ferrara et al., 2003). Additionally, VEGF-A stimulates the proliferation and induces chemotaxis of stalk cells during the initiation of branching angiogenesis up the concentration gradient (Klagsbrun et al., 1999; Dvorak et al., 2007). The amalgamation of tip and stalk cell processes creates a snail trail pattern of vascular formation as a new vessel is formed from stalk cells following the path of migration by the tip cells (Connor et al., 2015).

B) VEGF Receptors

There are three tyrosine kinase VEGFRs, VEGFR-1, VEGFR-2, and VEGFR-3 (Takahashi, 2011). As single-pass receptor tyrosine kinases, the binding of VEGF ligands to immunoglobulin domains on the extracellular region of VEGFR monomers prompts dimerization, activation, and trans-phosphorylation of VEGF receptors (von Tiedemann et al., 2002). Activation of VEGF signaling stimulates the production of platelet activating factor (PAF), increasing mitogenesis and permeability of the vasculature (Ogawa et al., 1998; Bernatchez et al., 2002). While VEGFR-1 has high affinity for VEGF-A ligand, it possesses weak kinase activity and may function as either a promoter or an inhibitor of angiogenesis by acting as a decoy receptor, competing with VEGFR-2 for VEGF-A binding (Hiratsuka et al., 1998; Meyer et al., 2005; Funahashi et al., 2010). VEGFR-2, on the other hand, has highly active kinase activity and is largely responsible for regulating cellular responses to VEGF-A (Yancopoulos et al, 2000; Rahimi, 2006).

C) Role of posttranslational modifications in regulation of VEGFRs and angiogenesis

Recent studies have revealed additional dimensions of regulation on the VEGF pathway in the form of posttranslational modification by glycosylation or phosphorylation and methylation (Rahimi and Costello, 2015). Extracellular glycosylation on the immunoglobulin domains of VEGFR-2 is important for ligand dependent activation for signal transduction (Takahashi et al., 1997; Chandler et al., 2016). On the intercellular region, the mode of modification is phosphorylation, which

occurs on tyrosine, serine, and threonine residues. A study done using single tyrosine mutations on VEGFR-2 found that phosphorylation of tyrosine Y1052 and Y1057 residues was required for kinase activity (Meyer et al., 2008). Furthermore, Y1173 phosphorylation modulates VEGFR-2 activity by recruiting important signaling factors such as PLCγ1 and p85 of PI3-kinase (Dayanir et al., 2001; Meyer et al., 2003). Another method of modulating VEGFR activity is through methylation of lysine (Lys) and arginine (Arg) residues (Rahimi and Costello, 2015). Methylation of Lys1041 on VEGFR-2 is necessary for regulating VEGFR-2 activation and angiogenesis (Hartsough et al., 2013).

Given the relatively low expression in typical adult tissue and significant involvement in tumor angiogenesis, VEGF as well as VEGFRs have been targets for treatment of angiogenesis related pathologies using inhibitors such as bevacisnub and sunitinib (Elice, et al., 2012).

Role of Ephrin receptors in angiogenesis

Eph receptor tyrosine kinases belong to the largest class of receptor tyrosine kinases, and are implicated in a diverse variety of cellular responses, such as cell adhesion, invasion, and repulsion (Poliakov et al., 2004). They also regulate vasculogenesis, angiogenesis, and axon pathfinding during embryonic development (Egea, 2007).

Ephs are divided into type A and B based on their association with ligands Ephrins A or B (Kullander and Klein, 2002). Both types conform to the typical structure

of receptor tyrosine kinases, which include an extracellular globulin ligand-binding domain, a transmembrane domain, and an intracellular kinase domain (Yancopoulos et al, 2000).

Similar to Eph receptors, Ephrin ligands are classified into Class A or Class B. Class A Ephrins preferentially binds EphA receptors and are anchored to the plasma membrane by glycosylphosphatidylinisotol (GPI) while class B ephrins differ in having a transmembrane domain and typically associating with EphB receptors (Yancopoulos et al, 2000. Despite this preference of receptor-ligand association within each class, there exists a high degree of promiscuity between the each class of Ephs and ephrins (Pasquale, 2004).

Ephrin/Eph pathway activation is unique among RTKs, which involves the formation of ligand clusters or multi-dimerization (Davis et al., 1994; Surawska et al., 2004). Furthermore, unlike the 'forward signaling' mechanism of most RTKs, the Eph/ephrin pathway signaling is bi-directional, meaning both Eph receptors and ephrin ligands are capable of generating cellular responses (Himanen and Nikolov, 2003).

Cellular responses elicited by Ephrin/ephs, such as cell motility and adherence, are key components in regulating angiogenesis, particularly in arterial and venous vessel formation. Of particular interest are ephrinB2 ligand and receptor EphB4, which are largely expressed in arterial endothelial cells and venous endothelium, respectively (Lawson, 2002; Kujiper et al., 2007). EphrinB2 and EphB4 are responsible for artery/venous differentiation early in development and are crucial in establishing

endothelial cell positioning for sprouting capillary growth during vessel remodeling (Wang et al., 1998).

Role of NOTCH pathway in angiogenesis

The NOTCH signaling pathway represents another fundamental regulator of angiogenesis. First identified in drosophila, the NOTCH signaling is a highly conserved pathway and central in cell differentiation and cell fate determination (Artavanis-Tsakonas et al., 1999; Greenwald et al., 1983). Knockout mice studies of the NOTCH signaling pathway have demonstrated that embryos lacking these receptors were incompatible with life, highlighting the importance of NOTCH expression during embryological development (Swiatek et al., 1994). Mammals possess four distinct NOTCH receptors, NOTCH1, NOTCH2, NOTCH3, and NOTCH4 (Radtke, 2003). NOTCH receptors are single-pass transmembrane proteins that differ in the number of EGF repeats on the extracellular domain and the presence of Transcription Activation Domains (TAD) and proline, glutamate, serine, and threonine (PEST) motifs within the intracellular domain (Radtke, 2003; Chillakuri et al., 2012; Olsauskas-Kuprys et al., 2013).

In mammals, NOTCH activation is initiated by 5 single pass transmembrane ligands, serrate-like ligands, known as Jagged, (JAG 1 and 2) and Delta-like ligands (Dll 1, 3, and 4) (Radtke, 2003). These ligands are noted for containing an N-terminal DSL (Delta, Serrate, Lag2) domain that contains a binding site for NOTCH (Tax et al., 1994; Chillakuri et al,. 2012).

A) Canonical NOTCH Signal Transduction

NOTCH ligands interact with the NOTCH receptors in trans between cells, leading to a sequence of cleavages culminating in a S3 proteolytic cleavage of the NOTCH intercellular domain (NICD) by gamma secretase (GS) and its release into the cytoplasm (Kopan and Ilagan, 2009). NICD is subsequently translocated into the nucleus where it associates with transcription factors and DNA binding proteins such as recombining binding protein suppressor of hairless (RBPJ or CBF-1) and Mastermind (MAML1), forming a transcriptional complex that activates target genes, such as HES/HRT (Hairy/Enhancer of Split genes) (Greenwald, 1998).

Ligands are also capable of inhibiting NOTCH signaling through autonomous *cis*-interactions between ligands and NOTCH on the same cell, though the mechanism of such interaction is not fully understood (Sakamoto et al., 2002; Ladi et al., 2005).

B) Role of NOTCH signaling in angiogenesis

The involvement of NOTCH signaling in angiogenesis is strongly supported by expression of NOTCH receptors and ligands in endothelial cells, particularly Dll4 expression, which is restricted to arterial endothelium (Shutter et al., 2000). Up-regulation of ligand Dll4 in response to VEGF signaling activates NOTCH pathway, inhibiting tip cell selection, endothelial cell migration, and angiogenesis (Suchting et al., 2007; Trinidade et al., 2008). Conversely, JAG 1 antagonizes the effects of Dll4/NOTCH signaling. Up-regulating JAG 1 increases sprouting angiogenesis and stability of capillary vessels (Benedito et al., 2009).

As with most biological processes, angiogenesis is a complex event regulated by the coordination between the multiple signaling pathways. Accordingly, NOTCH modulates angiogenesis through regulating VEGFR2 and Ephrin pathways (Liu et al., 2003; Lobov et al., 2007). Like VEGF, NOTCH signaling is also involved the tip cell determination. VEGF-A/VEGFR-2 signaling within a single endothelial cell increases its production of ligand Dll4, which interacts with NOTCH1 receptors on neighboring cells, causing a reduction in VEGFR-2 expression while simultaneously increasing VEGFR-1 expression (Jakobsson et al., 2010). By altering the ratios of VEGFR expression, NOTCH signaling decreases VEGF-A sensitivity among other endothelial cells , allowing just one endothelial cell with an up-regulation of VEGFR-2 and Dll4, and high sensitivity to VEGF-A to become a tip cell.

NOTCH also mediates vascular morphogenesis by regulating the expression of EphB4 and ephrin B2 (Heroult et al., 2006). Stimulation of NOTCH signaling by overexpressing Dll4 promotes arteriogenesis during vascular development in endothelial cells by up-regulating ephrin B2 (Duarte et al., 2004; Kofler et al., 2011) while simultaneously depressing expression of venous marker EphB4 (Trinidade et al., 2008).

Animal studies using zebrafish models have shown NOTCH pathway to exert negative regulation on capillary branching, making NOTCH regulation a potentially reliable target of treatment for cancers that are non-responsive to anti-VEGF treatments (Siekmann and Lawson, 2007; Suchting et al., 2007).s

C) The role of NOTCH in cancer development

A study published by Ellisen et al. (1991) was the first to reveal the involvement of NOTCH pathway with cancer in human T-ALL lymphoma. Now, it is well accepted that aberrant NOTCH signaling is involved in the regulation of key tumorigenic processes such as proliferation, epithelial to mesenchymal transition (EMT), and angiogenesis (Timmerman et al, 2004; Ridgeway et al., 2006). Paradoxically, it appears that NOTCH may act as both a promoter and tumor suppression even within the same tumor (Radtke, 2003; Leong et al., 2006).

Canonical Dll4/NOTCH signaling is commonly manipulated during the course of tumor development (Mailhos et al., 2001). Tumors heavily rely on vasculature as a resource for tumor expansion and network for metastasis (Cavallo et al., 1972; Folkman and Hanahan, 1991). During initial tumor growth, the release of tumor angiogenesis factors (TAF) from tumors and the creation of a hypoxic and nutrient-deprived environment stimulate persistent activation of VEGF and NOTCH pathways (Folkman et al., 1971; Ferrara, 2002; Kofler et al., 2011). This induces over-abundant vessel formation that is essential for tumor progression.

Studies into Dll4/NOTCH regulation of tumor angiogenesis discovered that blocking Dll4 interaction with NOTCH-2 with DLL-4 specific antibodies cause hyper-proliferation of endothelial cells that resulted in defective vasculature, affecting blood flow and thus inhibiting tumor growth (Ridgway et al., 2006).

Pathological Angiogenesis

Pathological angiogenesis is the dysregulation of angiogenic processes that is exemplified by deficient or excessive vessel formation. An overstimulation of angiogenesis is associated with human pathologies, including tumorigenesis and metastasis (Klagsbrun, 1991).

Tumor growth and angiogenesis are inter-dependent events that modulate each other (Warren and Shubik et al., 1966; Folkman, 1971). Studies conducted on tumorigenesis demonstrated that, not only are tumors able to recruit and induce proliferation of endothelial cells and vessel sprouting to the site of growth, solid tumors rely significantly on an established blood source to evolve past dormancy to a metastatic state (Cavallo et al., 1972; Greene, 1941; Holmgren et al., 1995). This critical relationship between angiogenesis and tumorigenesis has made anti-angiogenesis an exciting and, more importantly, specific area of study for therapeutics targeting tumor growth and metastasis (Kubota, 2012).

Identification of Major Intrinsically Notch-2 Associated Receptor (MINAR) as a novel regulator of angiogenesis

The purpose of this study was to elucidate the role of MINAR in angiogenesis. Originally named KIAA1024, Major Intrinsically-disordered Notch-2 Associated Receptor (MINAR) was first identified as one of 2031 novel cDNAs of the Human Unidentified Gene-Encoding (HUGE) protein database in an effort to characterize these proteins (Kikuno et al., 2004). Subsequently, MINAR was identified in our lab alongside

several other novel immunoglobulin containing cell surface proteins such as Immunoglobulin Proline-rich Receptor-1 (IGPR-1), TMIGD1, and Immunoglobulin Cysteine-rich Receptor-1 (IGCR-1) (Rahimi et al., 2012; Arafa et al., 2016; Wang et al., 2016). Found on human chromosome 15, this protein is composed of 916 amino acids and has a predicted molecular weight is 100kDa. MINAR was determined to be an intrinsically disordered novel cell surface receptor and a putative ligand for the NOTCH-2 receptor. MINAR was postulated to negatively regulate angiogenesis via the canonical NOTCH signaling pathway through direct association with NOTCH 2 receptor.

The specific goals of this project were as follows:

A) Test the hypothesis that MINAR is an intrinsically disordered protein.

B) Establish the role of MINAR in regulation of NOTCH-2 activity.

C) Elucidate the regulatory role of MINAR on NOTCH-2 mediated angiogenesis.

METHODS

Antibodies and Reagents

Rabbit polyclonal anti-MINAR antibody was developed in our laboratory and its specificity was further validated (unpublished data). Anti-MINAR antibody was used in 1:2,000 dilutions to detect for the presence of MINAR in PAE and HEK-293 cells in western blot analysis. Mouse monoclonal anti-GAPDH antibody (1:5000 dilution) and mouse polyclonal anti-alpha tubulin antibody (1:5000 dilution) were purchased from Santa-Cruz Biotechnology, Inc. Rabbit polyclonal anti-NOTCH2 antibody was purchased from Cell Signaling Technology.

Cell Culture

Human Embryonic Kidney cells (HEK-293) and Porcine Aortic Endothelial cells (PAE) were maintained in Dulbecco's Modified Eagle's Media (DMEM) containing 10% fetal bovine serum (FBS) and 50 units/mL Penicillin and Streptomycin. Cells were incubated at 37℃ in a 5% CO_2 humidified chamber.

Cell Transfection

HEK 293 cells were plated at 60% confluence at the time of transfection and media was aspirated and replaced with 2mL serum free DMEM. In an epi-tube, 3ug of MINAR shRNA control, 412, 615, or 719 plasmids or control shRNA, 9uL PEI (polyethylenimine), and 400uL serum free DMEM were incubated in the tissue culture

hood for 15 minutes before being added to the cells. Cells were then incubated for 6 hours before 2mL of DMEM 10% FBS was added to existing media.

After 24 hours, media in the plates were replaced with DMEM 10% FBS with puromycin to select for transfected cells. Some cells were lysed after 48 hours to check for the knock down of MINAR.

Retroviral virus production

HEK-293 GPG cells were grown to 90% confluence in GPG Growth Media before being transfected with shRNA MINAR using pGIPZ. After 24 hours, media was changed to Viral Producing Media and the virus was collected at 72, 96, 120, and 144 hours as described (Rahimi, et al., 2000, JBC). 4ml of virus was added to HEK-293 cells at 70% confluence on a 60mm plate along with 4ul of polybrene and left for 16-20 hours. DMEM 10% FBS with puromycin is added the next day to select for transduced cells.

Western Blot Analysis

Cells were rinsed twice with H/S (25mM Hepes (pH 7.4)/150mM NaCl) and collected in lyse buffer (10mM Tris-HCl, 10% Glycerol, 5 mM EDTA (pH 7.4), 50 mM NaCl, 50 mM NaF, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 20 mg/ml aprotinin). The resulting solution was centrifuged and supernatant was collected. 5X Sample Buffer (3.8% Tris-base, 50% glycerol, 5% sodium dodecyl sulphate (SDS), 5% β-mercaptoethanol, 0.0025% bromophenol blue) was added to whole cell lysates before being placed on heat block at 95℃ for 5 minutes. Cell lysate samples were

resolved on 10 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE) then transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with blotto (2% non-fat dry milk and 0.05% Tween-20 in Western Rinse) for 1 hour on a rocker. After washing with western rinse for 5 minutes, membranes were incubated with primary antibody for 1 hour at room temperature. Following three 10 minutes washes in western rinse, membranes were incubated with secondary antibodies for 1 hour. The membranes were washed again in western rinse three times, 10 minutes each.

4PBA Cell Culture Treatment and Trypsin digest

HEK-293 cells were incubated with 10mM PBA for 2 hours before being lysed. Cells designated for trypsin digest were lysed by mechanical homogenization using sonication with Thermo-Fisher Sonicator Dismembrator Model 1000. 20ng of trypsin was added to 50ul of whole cell lysate and was allowed to digest at room temperature for 0, 30, and 60 minutes. Following this, 5X sample buffer was added to lysates (1:5 dilution) and samples were denatured in the heat block at 95℃ for 5 minutes. Cell lysates were resolved on 10% SDS-PAGE and analyzed by Western Blot using anti-MINAR antibody.

Matrigel Capillary Formation Assay

The surface of each well in a 24 well plate was coated with 200 μL of Matrigel. PAE cells expressing pMSCV empty vector and MINAR (2×10^3 cells per well, triplicate wells per group) are seeded in each well and allowed to adhere for 1 hour. Each

cell line is then subjected to treatment with 500ul of GSI (purchased from Calbiochem). After 24 hours, images of the cell are captured using Zeiss microscope camera and Lumenera INFINITY ANALYZE Software. Formation of capillary tubes was analyzed with the Angiogenesis Analyzer via ImageJ software.

Reverse Transcription Quantitative Polymerase Chain Reaction Analysis of MINAR

TRIzol reagent (Life Technologies, Carlsbad, CA) was used to isolate total RNA. One microgram of total RNA from at least three mice per group was subjected to reverse transcription using High Capacity cDNA Reverse Transcription Kit (Life Technologies) according to manufacturer protocol. RT-qPCR was performed according to manufacturer recommendations (Applied Biosystems) and 18S housekeeping gene was used as internal controls.

RESULTS

MINAR is an intrinsically disordered protein

Our initial *in silico* analysis predicted MINAR as a putative cell surface protein with a single-pass transmembrane and a short cytoplasmic domain. The N-terminus extracellular domain of MINAR represents the largest portion of MINAR. Further examination using Metadisorder, an online program comprising 13 different protein disorder programs like DisEMBL, DISOPRED2, DISpro, Globplot, iPDA, IUPred, Pdisorder, Poodle-l, PrDOS, and Spritz, consistently predicted approximately 70% of MINAR is intrinsically disordered (Fig. 1) (unpublished data, Nader Rahimi, 2016).

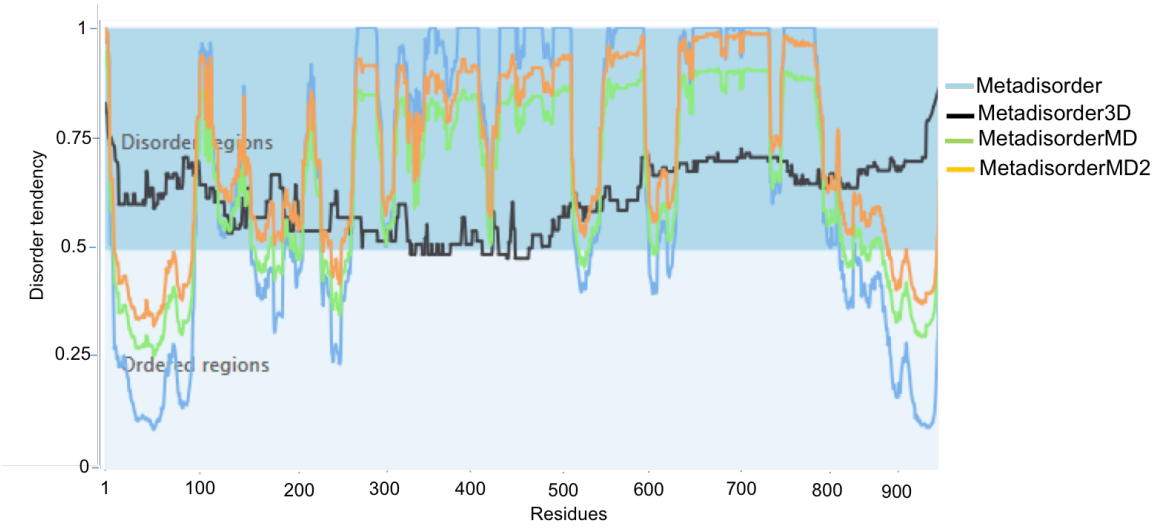

Figure 1. MINAR is predicted as intrinsically disordered. Metadisorder analysis predicted 70% of the MINAR sequence to be disordered. A disorder probability above 0.5 is considered disordered.

Considering the predicted intrinsically disordered nature of MINAR, we decided to explore its disordered characteristics experimentally. Accordingly, we hypothesized

that treatment of HEK-293 cells with 4-phenylbutyrate (4PBA), a well-characterized chemical chaperone should stabilize MINAR and increase its expression. On top of relieving protein aggregations, 4PBA is also known to reduce degradation of misfolded proteins by imparting stability to these proteins (de Almeida, 2007). Our preliminary Western Blot analysis showed that treatment of HEK-293 cells with 4PBA demonstrates increased expression of MINAR (Fig. 2a). Quantification of western blot revealed approximately a third more MINAR expression in 4PBA compared to untreated cells (Fig. 2b). The data suggests the idea of MINAR as an intrinsically disordered protein; however further studies are required to establish MINAR as an intrinsically disordered protein.

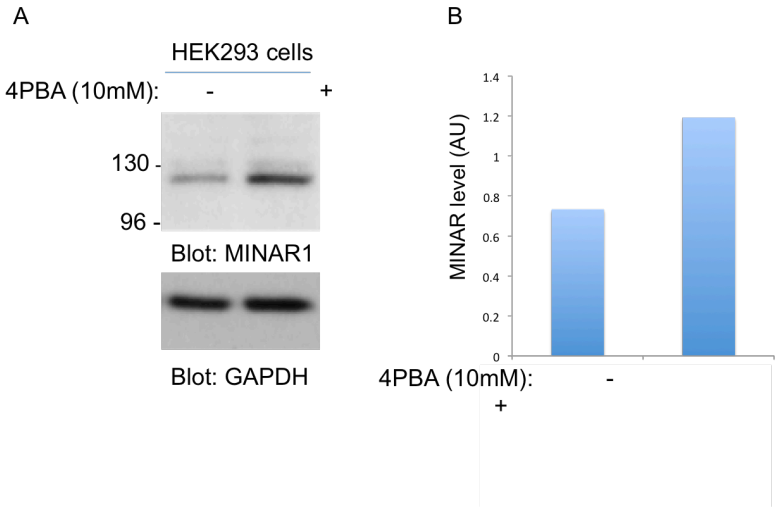

Figure 2. 4PBA increases expression of MINAR in HEK293 cells. A) HEK293 cells expressing endogenous levels of MINAR were treated with 10mM of 4PBA for 2 hours before being lysed. Whole cell lysates were blotted for MINAR. GAPDH was used as loading control. B) ImageJ quantification of MINAR expression between 4PBA treated and untreated groups from western blot analysis. Graph represents a single western blot.

Next, we examined whether the increased expression of MINAR in response to 4PBA treatment is associated with its increase stability and folding. We hypothesized that

if 4PBA treatment indeed improves MINAR folding and stability, the 4PBA treatment should decrease the susceptibility of MINAR to partial trypsin digest.

To this end, HEK-293 cells with and without 4PBA treatment were partially digested with 20ng of trypsin at room temperature for 0, 15, and 30 minutes. Western blot analysis showed that 4PBA treated cells was more resistant to the trypsin treatment, indicating that 4PBA treatment increased the folding of MINAR (Fig. 3a). The amount of MINAR detected over the time subjected with trypsin digest was quantified and normalized. The resulting graph depicted a decrease in rate of degradation from HEK293 cells treated with 4PBA (Fig. 3b). Taken together, the data suggests that the 4PBA treatment increases the folding of MINAR leading to the stabilization of its secondary structure, which reduces MINAR's susceptibility to degradation.

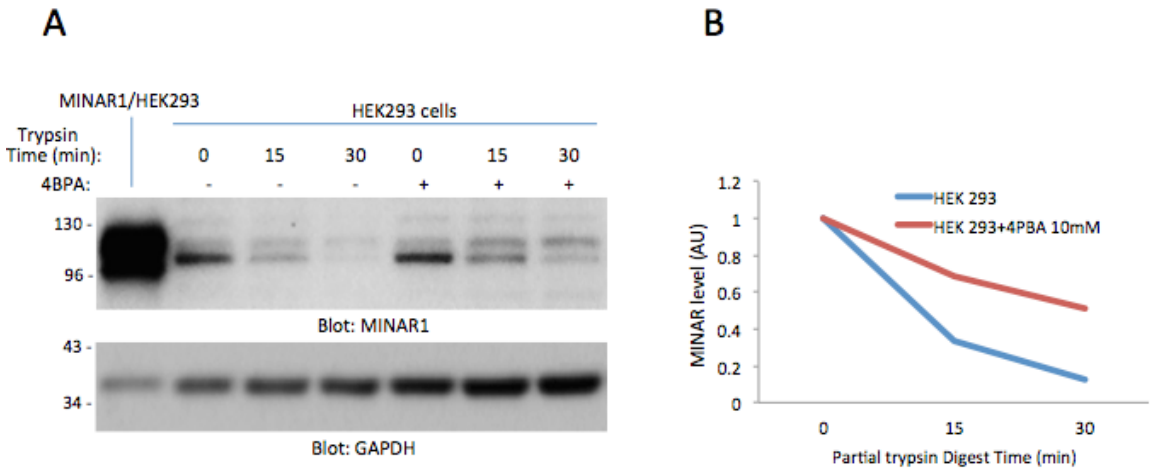

Figure 3. 4PBA reduces the rate of degradation of MINAR by trypsin. A) Untreated HEK293 cells and cells treated with 4PBA were partially digested by 20ng of trypsin for 0, 15, and 30 minutes before being blotted with anti-MINAR. GAPDH was used as loading control. B) ImageJ quantification of MINAR bands was plotted and normalized using MINAR/GAPDH.

Identification of NOTCH2 as MINAR binding protein

Given its large and disordered characteristics, we hypothesized that similar to other known intrinsically disordered proteins such as tumor suppressor p53, MINAR may interact with other proteins in order to gain structure and stability (Iakoucheva, 2002). We used liquid chromatography–tandem mass spectrometry (LC-MS/MS) to identify putative MINAR associated proteins. LC-MS/MS analysis identified NOTCH2 as a possible MINAR interacting protein (unpublished data, Kevin Chandler and Nader Rahimi, 2015) (Fig. 4).

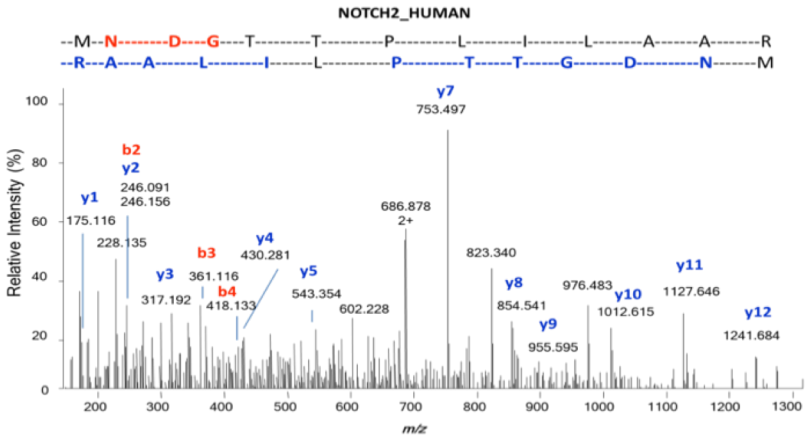

Figure 4. NOTCH2 is a putative binding target of MINAR. Mass spectrum analysis illustrates peptide sequence of human NOTCH2.

To validate the LC-MS/MS analysis findings, we performed a co-immunoprecipitation experiment, where MINAR was isolated from HUVEC (human umbilical vein endothelial cells) whole cell lysate through immunoprecipitation using anti-MINAR antibody and subjected to western blot analysis using anti-NOTCH2

antibody. The results illustrated that NOTCH2 was co-immunoprecipitated (co-ip) with MINAR (Fig. 5a). As a further proof of concept, the co-ip experiment was repeated with the inclusion of MINAR knock down HUVEC cell line. As seen in Fig.5b, western blot shows a weak band in lane 2 for NOTCH2 coinciding with the knock down of MINAR expression. This indicates a decrease in the amount of NOTCH2 co-immunoprecipitated with MINAR in the knock down cell line (unpublished data, Rosana Meyer, 2015).

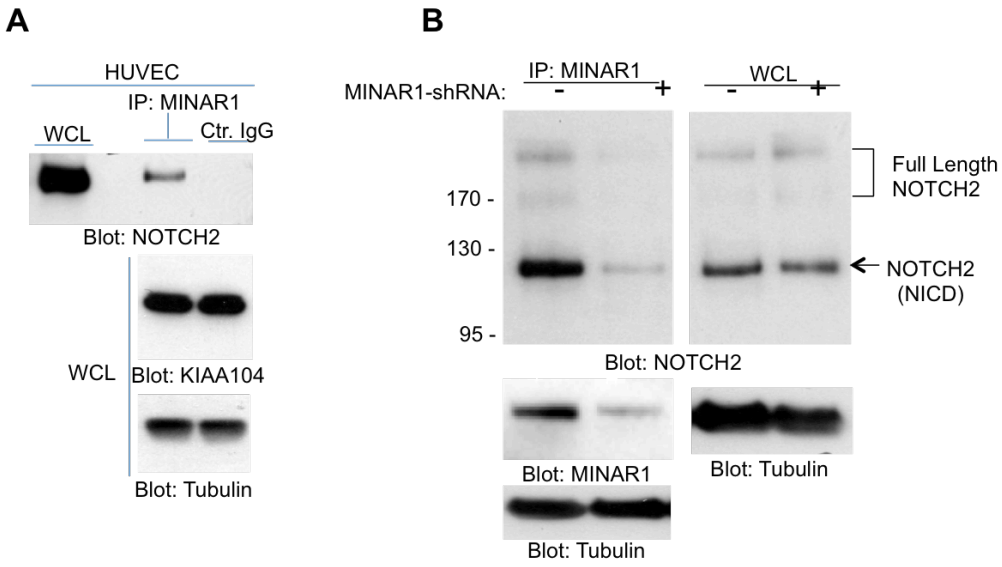

Figure 5. NOTCH2 is co-immunoprecipitated with MINAR. A) MINAR was immunoprecipitated from HUVEC whole cell lysate by anti-MINAR. The resulting lysate was run on western blot and blotted with NOTCH2 antibody. Alpha tubulin was used as loading control. B) MINAR was purified from shRNA knock down HUVEC whole cell lysate with MINAR antibody and run along side an shRNA control. Western blot was blotted with NOTCH2 and MINAR antibody. Alpha tubulin was used as loading control.

MINAR co-localizes with NOTCH2

In order to further examine the relationship between MINAR and NOTCH2, we studied whether MINAR co-localizes with NOTCH2 in cells. PAE cells were subjected to immunofluorescence microscopy to visualize the co-localization of MINAR with NOTCH2. The analysis demonstrated that MINAR and NOTCH2 are co-localized together at the cell surface of PAE cells (Fig. 6). Altogether, these experiments suggest that MINAR physically interacts with the NOTCH2.

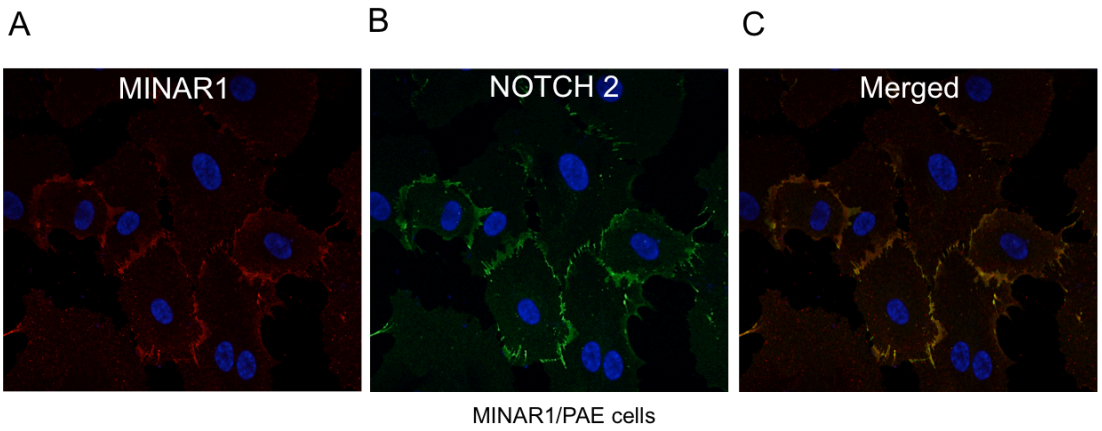

Figure 6. MINAR and NOTCH-2 co-localize at the cell surface. A) Immunofluorescence microscopy imaging of PAE cells depict MINAR at the cell surface. B) Immunofluorescence microscopy imaging of PAE cells depict NOTCH2 at the cell surface. C) Superimposition of A and B illustrate NOTCH2 and MINAR co-localized at the cell borders (unpublished data, Rosana Meyer and Nader Rahimi 2015).

MINAR is expressed in human blood vessels

To determine expression of MINAR in human tissues, we carried out qPCR and Western Blot analyses. Our analysis revealed the highest level of MINAR in the aorta, brain, and bone marrow (Fig. 7a). Subsequently, we performed immunohistochemistry

staining to determine which cell types MINAR is expressed in. MINAR was found specifically expressed in endothelial, aortic smooth muscle, and cytoskeletal muscle cells (Fig. 7b) (unpublished data, Rosana Meyer 2015). Taken together, the data demonstrate that MINAR is expressed in endothelial cells and associates with NOTCH2.

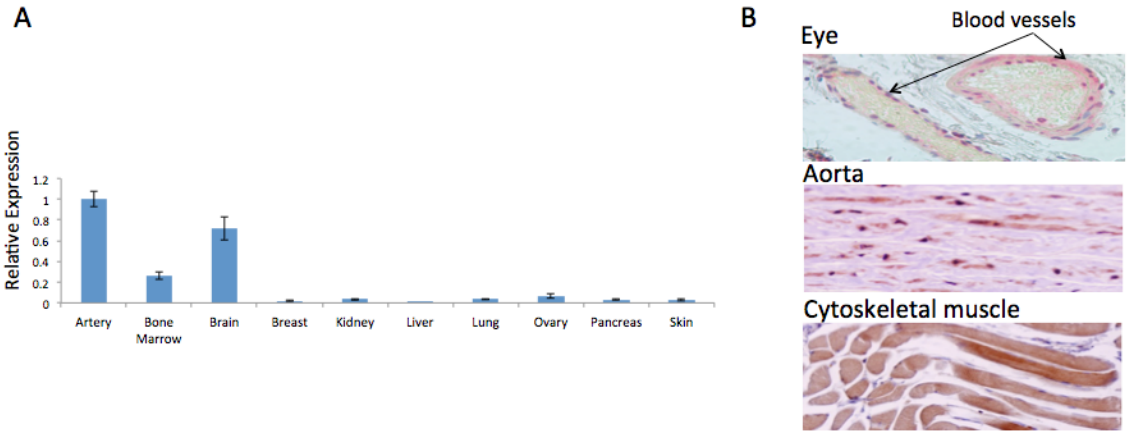

Figure 7. MINAR is expressed in human organs and tissues. A) qPCR analysis for relative expression of MINAR in human tissue is highest in the aorta, bone marrow, and brain. B) Immunohistochemical assay of human tissue suing anti-MINAR stains specifically for MINAR in endothelial, smooth, and cytoskeletal muscle cells.

MINAR interaction with NOTCH-2 potentially stimulates NOTCH2 activation

Experiments described above demonstrated that MINAR physically associates with NOTCH2. Therefore, we hypothesized that MINAR acts as a putative ligand to regulate NOTCH2 activation. To test our hypothesis, we knocked down MINAR with three different shRNAs and examined NOTCH2 activation. Expression of full length NOTCH 2 (200kD) and NICD (120kD), which corresponds to active NOTCH2 were compared between knockdown and endogenous MINAR groups. Western blot analysis

depicts HEK293 silenced for MINAR with a relatively darker band at 200kD and a lighter band at 120kD compared with shRNA control lysates, which had a relatively lighter band at 200kD and a stronger band lower down at 120kD. This subtle difference provides preliminary data that suggests diminished MINAR expression may correspond with a slight decrease in NOTCH2 activity. Additional rounds of shRNA MINAR knock down are required to optimize detection of the apparent affect of MINAR on NOTCH2 activity.

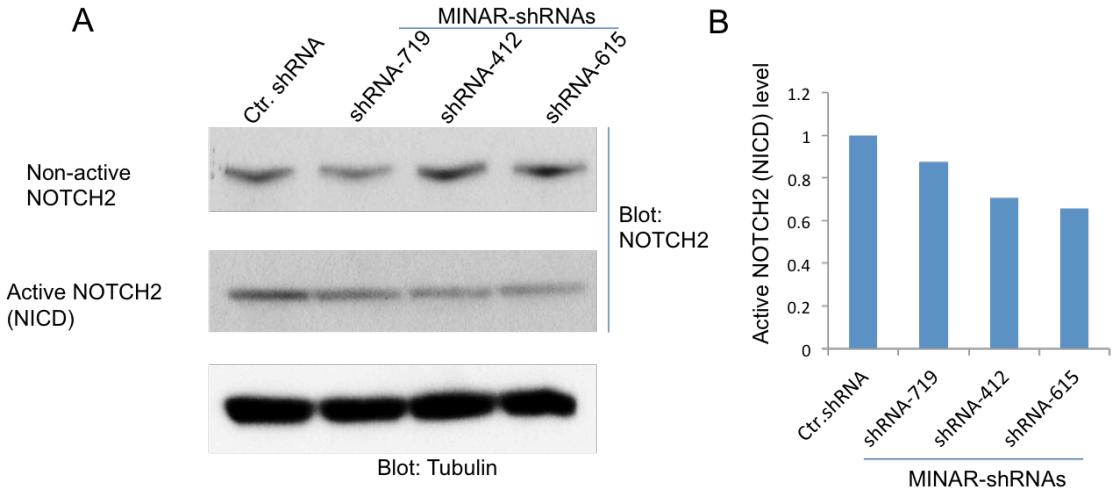

Figure 8. MINAR activates NOTCH2. A) Western Blot analysis of HEK293 knockdown MINAR cell lines and control shRNA were blotted for NOTCH2 and MINAR. Alpha tubulin was used as loading control. B) ImageJ quantification of NOTCH2 activation from western blot. Graph represents a single western blot.

MINAR inhibits angiogenesis

NOTCH activation is known to inhibit angiogenesis (Siekmann and Lawson, 2007). Accordingly, we hypothesized that MINAR interaction with NOTCH2 and subsequent NOTCH2 activation could inhibit angiogenesis. To explore the involvement of MINAR in angiogenesis, we studied capillary tube formation of PAE cells expressing

MINAR. The capillary tube formation of cells were observed under Zeiss microscope and images were captured using INFINITY ANALYZE software. The data showed that after 24 hours, capillary tube formation in the MINAR/PAE group was significantly decreased and were less extensive than the empty vector, or EV/PAE, control group (Fig. 8a-b). Additionally, capillaries formed in MINAR/PAE cells had more disruptions and showed signs of capillary retraction.

To provide further evidence that regulation of angiogenesis by MINAR is mediated through the NOTCH-2 pathway, we inhibited NOTCH-2 signaling by treating PAE cells with gamma secretase inhibitor (GSI) and NOTCH-2 blocking antibodies. GSI inhibits the S3 cleavage of NOTCH receptors and prevents the release the NICD while NOTCH-2 B9 antibody is expected to physically prevent MINAR/NOTCH-2 association (Olsauskas-Kuprys, 2003; Paris et al., 2005). Both MINAR/PAE cell groups treated with GSI and NOTCH-2 B9 antibodies demonstrated significantly more capillary branching than the control group, appearing to counter the inhibitory effect that MINAR exerts on angiogenesis (Fig. 8d). Interestingly, cells treated with NOTCH-2 B9 antibodies exhibited approximately twice as much capillary tubular length formation than cells with the GSI treatment (Fig. 8d). Taken together, the results indicate NOTCH-2 signaling is involved in the inhibition of capillary formation induced by MINAR.

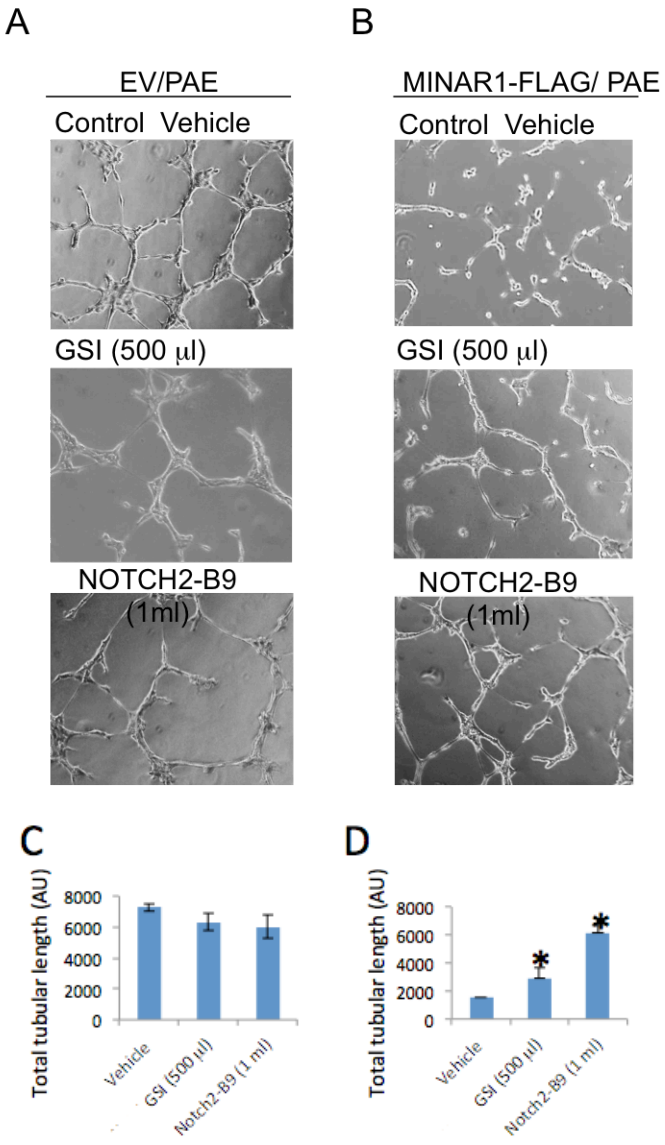

Figure 9. MINAR inhibits capillary tube formation. A) EV/PAE cells were grown on Matrigel and treated with GSI (500uL) and NOTCH2 blocking antibody (1ml). Images were captured after 24 hours. B) MINAR/PAE cells were grown in Matrigel and treated with GSI (500uL) and NOTCH2 blocking antibody (1ml). Images were captured after 24 hours. C) ImageJ quantification of total tubular length from EV/PAE cells using Angiogenesis analyzer. D) ImageJ quantification of total tubular length from MINAR/PAE cells using Angiogenesis analyzer.

DISCUSSION

In this study, we demonstrate that MINAR is a novel intrinsically disordered protein that interacts with NOTCH-2.

We used Metadisorder protein disorder program to analyze the intrinsically disordered nature of MINAR. MINAR was predicted to be a highly intrinsically disordered protein, with 70% of MINAR appearing to have no structure. The ordination of MINAR as an intrinsically disordered protein alludes to its key biochemical and possible biological function. For instance, a critical aspect behind the functionality of most intrinsically disordered proteins (IDP) is the dependence on other proteins to gain stability and order (Atkinson et al., 2016). While their lack of structure make IDPs flexible and accessible, the high degree of disorder means IDPs usually have a short half-life as they often form aggregations and are recognized by 20S proteasomes, resulting in degradation 'by default' (Tompa et al., 2008, Asher et al., 2006; Uversky; 2010). In this regard, MINAR is not expected to have a long half-life, unless it interacted with NOTCH2 or with other yet unknown proteins.

Considering IDPs gain some stability from forming secondary structures upon association with other proteins, we sought to elucidate the significance of structural stability on MINAR. Treating HEK293 cells with chemical chaperone 4PBA encouraged folding in MINAR, which concealed recognition sites and thus improved the stability of MINAR. This gain of order not only accounts for the increased expression, but also reduced susceptibility of MINAR to degradation upon trypsin digestion. Results from mass spectrometry, *in vivo* co-localization study, and co-immunoprecipitation assays all

revealed a physical interaction between NOTCH-2 and MINAR. Taken together, these observations underscore the critical role of NOTCH2 in governing the expression and possible function of MINAR. In this regard, NOTCH2 acts to safeguard MINAR from degradation, which represents an intriguing characteristic of MINAR.

Another important aspect of this study is the demonstration that MINAR is expressed in human blood vessels and endothelial cells, raising a possibility that MINAR could play a role in angiogenesis and other endothelial functions. Our studies revealed that MINAR plays a critical role in angiogenesis by modulating capillary tube formation of endothelial cells. Over-expression of MINAR inhibited capillary tube formation in PAE cells. Intriguingly, the observed effect of MINAR in capillary tube formation of endothelial cells is similar to that of NOTCH pathway, which is congruent with our hypothesis that MINAR activation of NOTCH2 would inhibit angiogenesis. In support of possible MINAR/NOTCH2 axis in the regulation of angiogenesis, preventing the release of the NOTCH Intracellular Domain (NICD) with either GSI or NOTCH-2 B9 antibody impeded normal anti-angiogenic effect of MINAR. This observation further signifies that MINAR plays a role in inhibiting angiogenesis and that the NOTCH-2 signaling pathway mediates the anti-angiogenesis effects observed.

The involvement of MINAR in regulating NOTCH2 activation and angiogenesis pegs it as a potential site of anti-angiogenesis or anti-cancer therapy. Targeting pathologic angiogenesis, either insufficient (*e.g*. ischemic cardiovascular diseases) or excessive angiogenesis (*e.g*. cancer & others) is a promising strategy that could revolutionize treatment of human diseases ranging from peripheral artery disease to

diabetic retinopathy and cancer. Current strategies mainly targets vascular endothelial growth factor (VEGF) pathway. Although they confer clinical benefits, their successes are restricted by insufficient efficacy, refractory or development of resistance (Ferrara and Kerbel, 2005; Fukumura and Jain, 2012). Cardiovascular disease is the leading cause of death in US and is associated with occlusion of the blood vessel in the affected organs. Restoring blood supply through therapeutic angiogenesis, in effect stimulating the growth of new blood vessels, is a tantalizing strategy that could lead to the successful treatment of cardiovascular diseases (Deveza et al., 2012). Thus, comprehensive insight into the regulation of vascular growth for the resolution of a particular pathology can bring indisputable therapeutic value to a diverse range of human pathologies. Similarly, the application of tissue engineering for organ regeneration and wound healing requires a more extensive understanding of vessel stabilization and growth.

Considering the clinical importance and therapeutic potential of modulating vascular network in various diseases, further investigation into the functional importance of MINAR and mechanism of its action in normal and pathological circumstances, could grant further insights into the mechanisms of angiogenesis and possible novel therapeutics.

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